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13. ABSTRACT (Maximum 200 Words)

The sphingolipid metabolite sphingosine-1-phosphate (S1P) is the ligand for a family of five specific G protein-coupled receptors ($S1P_{1-5}$) that regulate a wide variety of important cellular functions, including growth, survival, and cell motility. However, whether it also has an intracellular action is still a matter of debate. S1P is formed by the ATP-dependent phosphorylation of sphingosine catalyzed by types 1 and 2 sphingosine kinase (SphK). Many studies have shown that SphK1 stimulates cell proliferation and protects cells from apoptosis. In contrast, expression of SphK2 inhibits growth and enhances apoptosis independently of S1P receptor activation. We investigated the role of SphK2 in apoptosis of human breast carcinoma MCF7 cells in response to the DNA damaging agent doxorubicin. Expression of SphK2, which is predominantly localized to the nucleus of MCF7 cells, enhanced apoptosis induced by doxorubicin. Expression of SphK2 also significantly increased beta-galactosidase activity, a marker of senescence. However, in contrast to doxorubicin which increases the cyclin-dependent kinase inhibitor $P21^{WAF1}$ and p53 levels, SphK2 expression increased p53-independent expression of $P21^{WAF1}$ and hypophosphorylation of the retinoblastoma protein (pRb). Importantly, down-regulation of endogenous SphK2 protected MCF7 cells from doxorubicin-induced apoptosis and its effects on p21 without affecting p53.

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INTRODUCTION

Sphingolipid metabolism is a dynamic and tightly regulated process resulting in the formation of a number of bioactive metabolites, including ceramide, sphingosine and S1P, which have all been implicated as important components of cell fate decisions (1,2). Ceramide and sphingosine are usually associated with apoptosis and negative effects on cell growth and survival, whereas S1P, formed by SphK1 opposes these effects. Increased ceramide and sphingosine levels have been shown to induce apoptosis in many cell types: for example, ceramide and sphingosine enhance apoptosis of radiation resistant prostate and breast cancer cells (3). In contrast, it has been shown that exogenous addition of S1P protects oocytes *in vivo* from radiation-induced apoptosis (4). This has led to the proposal that the balance between the cellular concentrations of
ceramide and sphingosine versus S1P, the "sphingolipid rheostat", is important in determining whether cells survive or die (5). In agreement, studies from our lab show that sphingosine is involved in mitochondria-mediated apoptotic signaling induced by doxorubicin in human breast cancer cells (6). Whereas, in sharp contrast, S1P, formed by phosphorylation of sphingosine protects against ceramide-mediated apoptosis and promotes estrogen-dependent tumorogenesis of MCF7 cells (7). Recently, another mammalian isoform of SphK (SphK2) was cloned and characterized in our laboratory (8). Although highly similar in amino acid composition and sequence, SphK2 is much larger than SphK1 and diverges in its amino terminus and central region. Although little is yet known about the functions of SphK2, these distinct differences imply that SphK1 and SphK2 may have different physiological functions (8). Indeed, rather than promoting growth and survival, SphK2 expression suppressed growth and enhanced apoptosis that was preceded by cytochrome c release and activation of caspase-3 (9,10). Moreover, SphK2-induced apoptosis was independent of activation of S1P receptors (9). However, currently little information exists regarding the apoptotic effect of SphK2 expression in breast cancer cell lines in response to anti-cancer agents. The anthracyclin doxorubicin, a major anti-tumor agent used for the treatment of a variety of human cancers including breast cancer, is known to cause cellular damage through a number of mechanisms including free radical formation and inhibition of topoisomerase II (11). $p21^{WAF1}$, a member of a family of cyclin dependent kinase (cdk) inhibitors, which also include p27 and p53, appears to be a major determinant of cell fate in response to anticancer therapy (12). When induced in response to various stimuli, $p21^{WAF1}$ expression results in the inhibition of G1 cyclin/cdk complexes and G1 arrest (13). This effect has been related, at least in part, to dephosphorylation of pRb, which then binds to and inactivates the transcription factor E2F (14). Both p53-dependent and –independent pathways of $p21^{WAF1}$ induction in cells exposed to DNA damaging and maturation/differentiation inducing agents have been described (15-17). The goal of the present investigation was to determine the effect of SphK2 expression on survival and apoptosis of MCF7 cells in response to doxorubicin.

BODY

Specific Aim 1. To examine the role of the BH3 domain of SphK and its interactions with Bcl-2 family members in human breast cancer cells.

Many studies have shown that SphK1 promotes cell growth and inhibits apoptosis (5). In contrast, we have recently reported that SphK2 does the opposite, inducing apoptosis in response to serum-deprivation (9). As part of the proposed specific aim 1 the effects of SphK2 expression were examined in MCF7 cells in response to doxorubicin (1 μg/ml). The results are shown in Figure 1A. Whereas, doxorubicin exposure exhibited minimal toxicity to cells cultured in the absence of serum, transient expression of SphK2 resulted in a pronounced increase in apoptosis. Importantly, downregulating expression of SphK2 using psilencer-SphK2 (Figure 1C) resulted in a pronounced decrease in apoptosis in MCF7 cells upon exposure to doxorubicin under conditions of serum deprivation (Figure 1B).

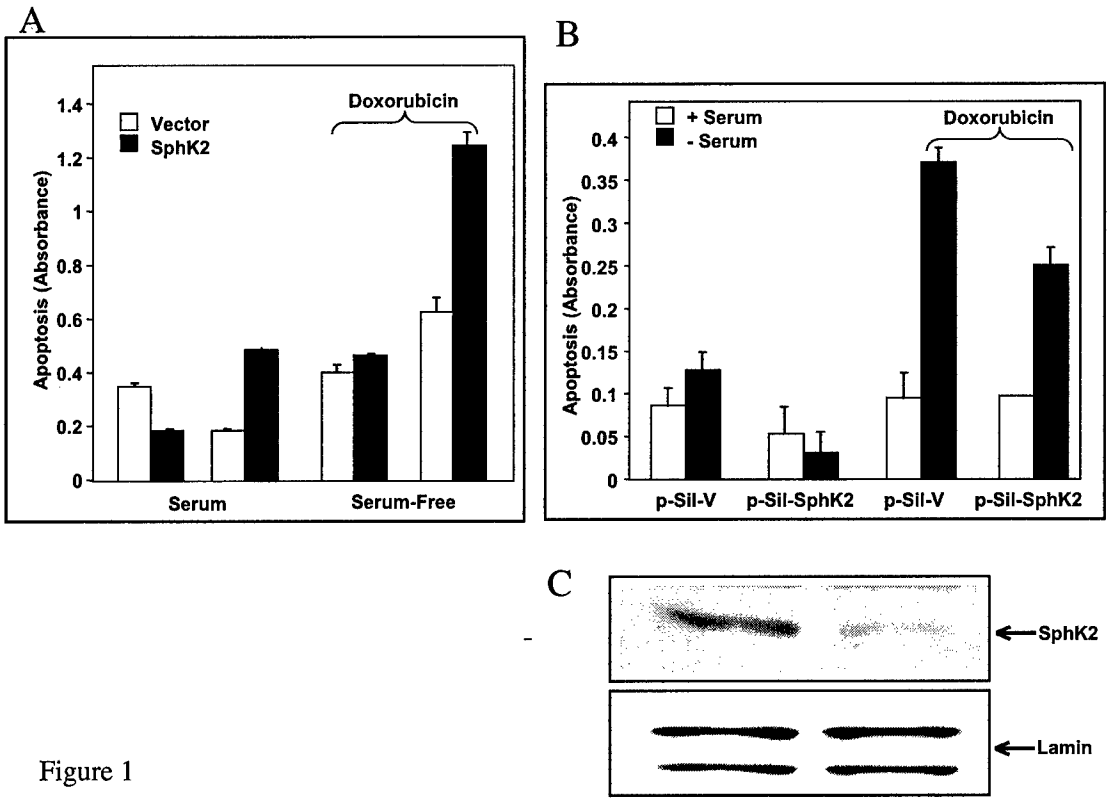

Figure 1

Figure 1. Effect of SphK2 expression on apoptosis of MCF7 cells. (A) Cells transiently transfected with vector or SphK2 were cultured in the presence or absence of doxorubicin in the absence or presence of 10% FBS for 24 h. Apoptosis was measured using a Cell Death Detection ELISA^{PLUS} kit. (B) MCF7 cells were transiently transfected with p-Silencer vector or p-Silencer SphK2, treated as in (A) and apoptosis measured. (C) MCF7 cells were transfected with pSilencer-vector and pSilencer-SphK2 and after 48 h, nuclear proteins were isolated as described in Materials and Methods. Equal amounts of protein were resolved by SDS-PAGE and transblotted to nitrocellulose. After incubation with anti-SphK2 antibody, immunopositive bands were visualized by enhanced chemiluminescence. The blot was stripped and re-probed with anti-lamin antibody as a loading control.

Specific Aim 2. To determine the role of caspase cleavage of the DxxD motif of SphK2.

Previously truncated forms of SphK2 split after its BH3 domain into a 227 amino acid N-terminal fragment and a 391 amino acid C-terminal fragmentin NIH 3T3 cells. Only the N-terminal fragment , which contains the putative BH3 domain induced apoptosis while the C-terminal fragment did not. Interestingly, these fragments would potentially be produced by caspase cleavage at the $D^{224}XXD^{227}$caspase recognition sequence in SphK2. Additionally, upon treatment with doxorubicin (1μg/ml) initial results show that endogenous SphK2 levels are reduced in MCF7 cells leading to the hypothesis that SphK2 is cleaved. However initial results with pre-treatment with the pan-caspase inhibitor z-VAD-FMK still resulted in reduced protein levels of SphK2. Further experiments need to be carried out to confirm this. The effects of proteosome inhibitors and calpeptin (a calpepin inhibitor) on SphK2 cleavage will also be investigated.

Specific Aim 3. To examine the role of the catalytic activity of SphK2 in apoptosis of breast cancer cells.

As part of specific aim 3 it was proposed to examine the localization of SphK2 by confocal microscopy and subcellular fractionation. Previous data suggests that SphK1 is mainly cytosolic (19). However, the localization of SphK2 is still not clear and it has been suggested to be cell type dependent (10). When nuclear and cytoplasmic fractions of MCF7 cells were separated and its intracellular distribution was examined using a specific antibody raised against a peptide sequence unique to SphK2, nuclear localization of endogenous SphK2 was observed (Figure 2A). In agreement, confocal microscopy revealed that when V5 tagged-SphK2 was transiently expressed in MCF7 cells, it was mainly localized in the nuclei and to a lesser extent in the cytoplasm (Figure 2B and C), as determined by immunostaining with anti-V5 antibody.

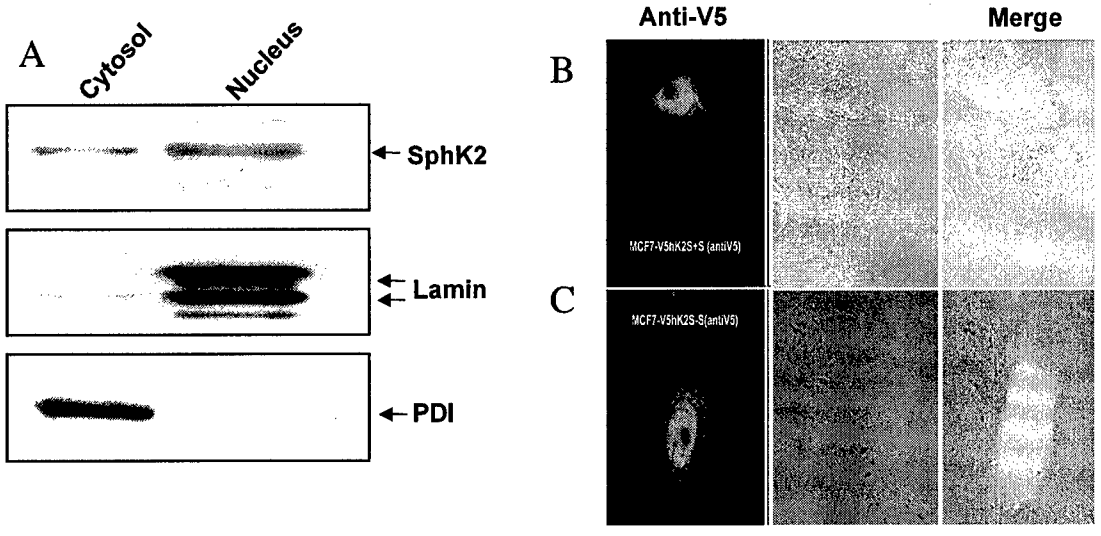

Figure 2

Figure 2. Nuclear localization of SphK2 in MCF7 cells. (A) Nuclear and cytosolic fractions were isolated and resolved by SDS-PAGE and then immunoblotted with an antibody directed toward SphK2. Antibodies for lamin A/C and PDI were used as nuclear and endoplasmic reticulum markers respectively. (B,C) MCF7 cells transfected with vector or V5-SphK2 were cultured for 24 h in the presence (B) or absence (C) of serum and SphK2 localization examined by confocal microscopy after staining with anti-V5 antibodies. (

Aim 4. To determine the role of endogenous SphK2 on apoptosis of breast cancer.
Previous studies have demonstrated the importance of $p21^{WAF1}$ in response to anti-cancer treatments (21,22). Therefore the effects of SphK2 expression on doxorubicin-mediated induction of $p21^{WAF1}$ were examined (Figure 3A). SphK2 expression had no effect on the upregulation of $p21^{WAF1}$ upon exposure to doxorubicin. However, modest upregulation was seen after transient transfection of SphK2 in MCF7 cells compared to transient transfection of an empty-vector plasmid. Similarly, underphosphorylated pRb was increased after transient transfection of SphK2 in MCF7 cells compared to transient transfection of an empty-vector plasmid. In contrast to the upregulation of $p21^{WAF1}$ expression by doxorubicin, which has been reported to increase $p21^{WAF1}$ in a p53-dependent manner (21), the increase in $p21^{WAF1}$ levels by SphK2 expression appears to be p53 independent (Figure 3A). Interestingly, reducing endogenous levels of SphK2 using pSilencer-SphK2 (Figure 1C) resulted in a decrease in $p21^{WAF1}$ protein levels in MCF7 cells upon exposure to doxorubicin under conditions of serum deprivation (Figure 3B). As expected, in contrast to expression of SphK2, transient transfection of SphK1 had no effect on $p21^{WAF1}$ expression (Figure 3C).

A biological phenomenon that involves $p21^{WAF1}$ is cell senescence, the physiological process of terminal growth arrest, accompanied by specific changes in cell shape, adhesion and gene expression (23). Breast

tumor cells can also be induced to undergo senescence through the overexpression of several tumor suppressors (24-26) including p21 (27,28) or through inhibition of oncogenes (29). Furthermore, it has been demonstrated that the growth arrested state associated with acute exposure to doxorubicin treatment of MCF7 cells results in the induction of a senescent phenotype (30). Therefore, we investigated whether expression of SphK2 has an effect on senescence under similar conditions. Following acute exposure (2 h) of MCF7 cells to 1 μg/ml doxorubicin, β-galactosidase expression, a marker of cellular senescence, was markedly increased 2 days after exposure. Transient transfection with SphK2 significantly increased the percent of cells that were β-galactosidase positive. As part of this specific aim the effects of endogenous Sphk2 on senescence of MCF7 cells in response to doxorubicin treatment will be investigated by reducing endogenous levels of SphK2 using p-silencer SphK2.

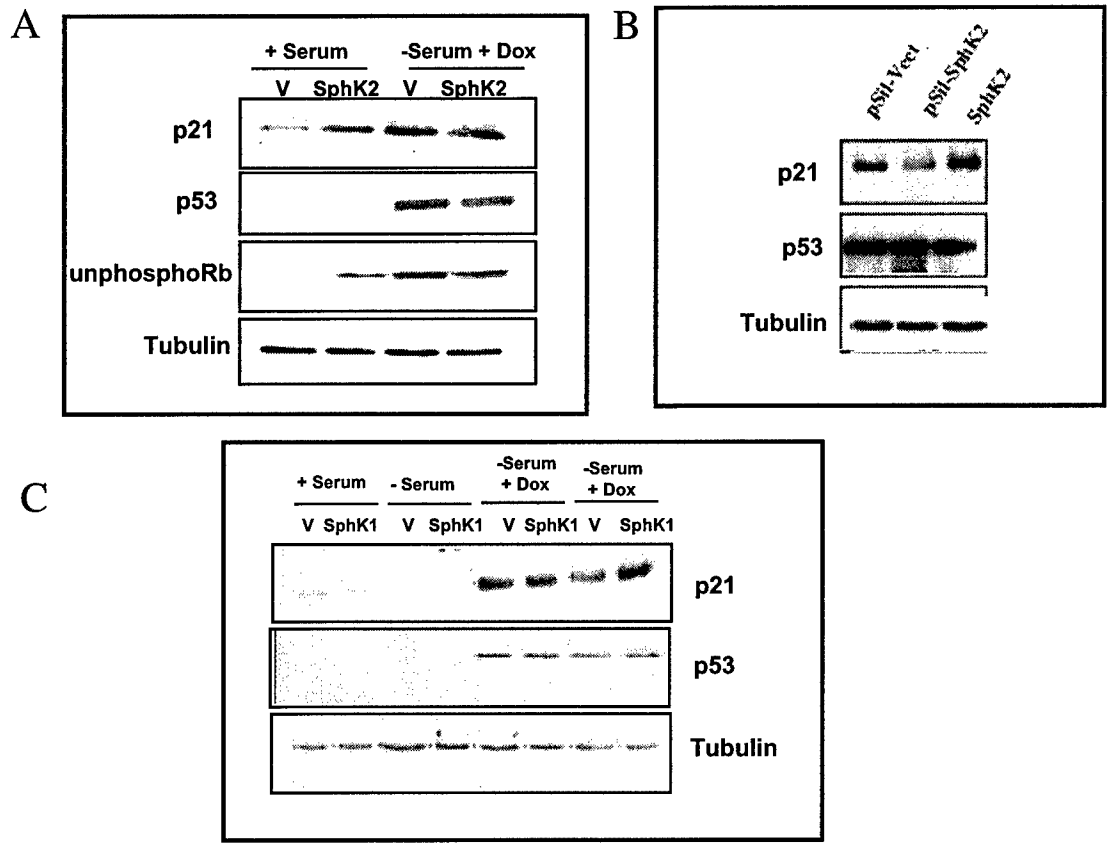

Figure 3.

Figure 3. Effect of SphK2 expression on doxorubicin-induced increases of $p21^{WAF1}$, underphosphorylated Rb, and p53. (A) MCF7 cells transiently transfected with vector or SphK2 were cultured in the presence or absence of doxorubicin (1 μg/ml) in 10% FBS or serum-free medium for 24 h. Cell lysates were resolved by SDS-PAGE and then immunoblotted with antibodies to underphosphorylated Rb, p21, and p53. Membranes were subsequently stripped and probed with tubulin antibody to show equal loading. (B) MCF7 cells were transiently transfected with p-Silencer vector or p-Silencer SphK2 and after 24 h, cells were treated with doxorubicin in serum-free media for 24 h. Cell lysates were resolved by SDS-PAGE and then immunoblotted with antibodies to p21 and p53.

Membranes were subsequently stripped and probed with tubulin antibody to show equal loading. (C) MCF7 cells transiently transfected with vector or SphK1 were cultured in the presence or absence of doxorubicin (1 μg/ml) in 10% FBS or serum-free medium for 24 h. Cell lysates were resolved by SDS-PAGE and then immunoblotted with antibodies to p21 and p53. Membranes were probed with tubulin antibody to show equal loading.

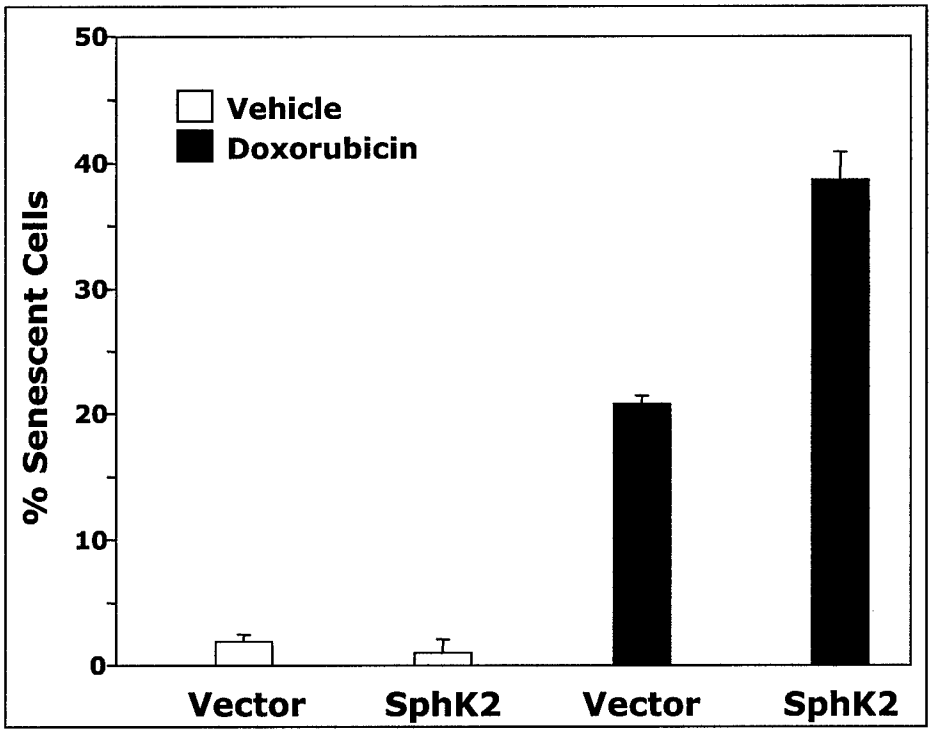

Figure 4

Figure 4. SphK2 expression increases senescence of MCF7 cells following acute exposure to doxorubicin. MCF7 cells transiently transfected with vector or SphK2 were treated in the presence or absence of doxorubicin for 2 hours. β-galactosidase expression was assessed 2 days after doxorubicin treatment. The percentage of positively stained cells was determined by counting three random fields of greater than 100 cells each.

Materials and Methods

Reagents

Doxorubicin was purchased from Sigma, reconstituted in molecular biology grade water, and stored protected from light as aliquots until dilution in culture medium immediately before cell treatments. Serum and medium were from Biofluids (Rockville, MD). X-gal was obtained from Gold Biotechnology

(St Louis, MO). Antibodies to $p21^{WAF1}$ and lamin A/C antibodies were purchased from Cell Signaling (Beverly, MA). p53 antibody was from Oncogene (San Diego, CA). Under-phosphorylated Rb antibody was from Transduction Laboratories (San Diego, CA). PDI antibody was from Stressgen Biotechnologies (San Diego, CA). Tubulin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). SphK2 rabbit polyclonal antibodies raised against a unique SphK2 peptide sequence (QALHIQRLRPKPEARPR) conjugated to keyhole limpet hemocyanin were generated by Biosynthesis Inc. (Lewisville, TX). SphK2 antiserum was purified on a protein A column followed by affinity purification on a Sulfolink gel conjugated with the same oligopeptide according to the manufacturer's instructions (Pierce, Rockford, IL). Secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

Cell Culture

MCF7 cells were cultured in phenol-red free IMEM supplemented with 4.5% glucose and 10% heat-inactivated FBS.

Down-Regulation of Endogenous SphK2

SphK2 expression was downregulated with sequence specific pSilencer-SphK2 designed according to the pSilencer user website (http://www.ambion.com). Two complementary oligonucleotides were synthesized (Invitrogen. Carlsbad, CA): sense, 5'-GATCCCGCTGGGCTGTCCTTCAACCTTCAAGAGAGGTTGAAGGACAGCCCAGCTTTTTTGGAAA-3'; antisense, 5'-AGCTTTTCCAAAAAAGCTGGGCTGTCCTTCAACCTCTCTTGAAGGTTGAAGGACAGCCCAGCGG-3'. Olgonucleotides were annealed and ligated into pSilencer vector according to the manufacturer's protocol (Ambion, Austin, TX). Cells in 10 cm dishes were transfected with pSilencer vector or pSilencer-SphK2 (4 μg) using Lipofectamine PLUS reagent (Invitrogen, Carlsbad, CA).

Nuclear Extract Isolation

Cytoplasmic and nuclear fractions were isolated from MCF7 cells using the NE-PER nuclear and cytoplasmic isolation kit (Pierce) according to the manufacturers instructions.

Western Analysis.

Cells were scraped in lysis buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 2 mM sodium orthovanadate, 4 mM sodium pyrophosphate, 100 mM NaF, 1:500 protease inhibitor mixture (Sigma). Equal amounts of proteins were separated by SDS-PAGE and transblotted to nitrocellulose, blocked with 5% non-fat dry milk for 2 h at room temperature and then incubated with primary antibodies overnight. Appropriate horseradish peroxidase-conjugated secondary antibodies were added in Tris-buffered saline containing 5% milk and immunoreactive signals were visualized by enhanced chemiluminescence (Pierce) and exposed to Kodak X-omat film.

Cell Death Assay.

Cell death was measured with the Cell Death Detection ELISA PLUS kit (Roche Diagnostics, Indianapolis, IN) that determines cytoplasmic histone-associated fragments (mononucleosomes and oligonucleosomes) as described previously (18). Alternatively, in some experiments, apoptotic cell death was measured by staining cell nuclei with the Hoechst dye bisbenzimide and apoptotic cells were identified by condensed, fragmented nuclear regions as previously described (19). A minimum of 300 cells were scored.

Immunofluorescence and Confocal Microscopy

MCF7 cells were grown on four-chambered slides (Nalge/Nunc) and transfected with SphK2 plasmid. Subcellular localization studies using confocal microscopy were performed as described previously with anti-V5 antibodies as a primary antibody and rabbit-FITC conjugate as a secondary antibody (10). Briefly, cells were washed with PBS, fixed for 20 min at room temperature with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. After washing, cells were incubated for 45 min with primary antibodies in PBS containing 0.1% BSA, and then for 45 min with the corresponding secondary antibodies conjugated with FITC. Coverslips were mounted on glass slides using an Anti-Fade kit and examined by confocal microscopy.

Beta-galactosidase Histochemical Staining

After treatment, cells were washed twice with PBS and fixed with 2% formaldehyde for 5 min. The cells were then washed again with PBS and stained with a solution containing 1 mg/ml 5-bromo-4-chloro-3-inolyl-β-galactosidase in dimethylformamide, 5 mM potassium ferrcyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 40 mM citric acid/sodium phosphate, pH 6.0, and 2 mM $MgCl_2$ (20). Following overnight incubation at 37 °C, the cells were washed twice with PBS. The percentage of positively stained cells was determined after counting three random fields of at least 100 cells each.

KEY RESEARCH ACCOMPLISHMENTS

(1) SphK2 promotes apoptosis in MCF7 cells.

(2) SphK2 is found to be predominantly localized to the nucleus in MCF7 cells.

(3) SphK2 Regulates p21 in MCF7 Cells.

(4) SphK2 expression increases senescence of MCF7 cells following acute exposure to doxorubicin.

REPORTABLE OUTCOMES

Abstracts

Sankala H, Hait N, Milstien S and Spiegel S (2004). The role of sphingosine kinase 2 in apoptosis. *Virginia Commonwealth University Massey Cancer Center 2004 Research Retreat*

Sankala H, Hait N, Milstien S and Spiegel S (2004). Sphingosine kinase 2 regulates apoptosis in human breast cancer cells. *The twenty-first annual Daniel T. Watts research poster symposium*

CONCLUSIONS

Since it is well established that S1P produced by SphK1 promotes cell growth and inhibits apoptosis, in part due to antagonism of ceramide-induced apoptosis (32,33), it was surprising to discover that a second isoenzyme, SphK2, was associated with inhibition of cell growth and was pro-apoptotic (9,10). Although both ceramide and S1P have emerged as key regulators of cell fate, studies examining the role of SphK2 in response to DNA damaging agents are lacking. The goal of the present investigation was to determine the effect of SphK2 expression in response to doxorubicin in MCF7 cells. Our results indicate that expression of SphK2 enhances apoptosis induced by doxorubicin, whereas, down-regulation of endogenous SphK2 protects against doxorubicin-induced apoptosis. These results support our previous observations that SphK2 promotes apoptosis (9). Interestingly, expression of SphK2 also significantly increased beta-galactosidase activity, a marker of senescence. However, in contrast to doxorubicin, which increases cyclin-dependent kinase inhibitor $P21^{WAF1}$ and p53 levels, SphK2 expression increased p53-independent expression of $P21^{WAF1}$ and hypophosphorylation of pRb. Importantly, downregulation of endogenous SphK2 protected MCF7 cells from doxorubicin-induced p21 upregulation without affecting p53. Recent evidence suggests that apoptosis and senescence may represent reciprocally regulated and mutually exclusive responses to chemotherapeutics (18). Our results indicate that SphK2 may be a critical component not only in apoptotic cell death but also in senescence associated with acute doxorubicin treatment. These findings raise interesting questions as to how SphK2 regulates p21 expression. Both p21 and pRb function as key regulators of the cell cycle, and evidence has been provided for the ability of ceramide to regulate both p21 and pRb (34-36). Over the past few years, there has been escalating interest in the role of ceramide and its metabolites in normal physiology and pathology. Levels of ceramide can be modified by various biological response modifiers and drugs (37). Interestingly, results from our laboratory have shown that expression SphK1 reduces ceramide accumulation and conversely, expression of SphK2 increases ceramide accumulation in HEK 293 cells (Maceyka, unpublished). Advancement in defining sphingolipid-dependent signaling pathways, derived from establishing a large body of biochemical, genetic, and physiological data, may provide new targets for modulation of anti-cancer drug responses with important potential for clinical applications in breast cancer.

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